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Surface binding and internalization of platelet-derived growth factor in human fibroblasts

(cell culture/endocytosis/electron microscopic autoradiography/indirect immunofluorescence)

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ABSTRACT Surface binding and uptake of platelet-derived growth factor (PDGF) in human fibroblasts cultivated *in vitro* were studied by quantitative electron microscopic autoradiography using ^{125}I-labeled PDGF and by indirect immunofluorescence using PDGF antibodies. After 120 min at 12°C PDGF was found preferentially in coated regions of the plasma membrane. Warming the cells to 37°C initiated rapid ingestion of the factor via small vesicles, usually lacking a membrane coat. After 10 min PDGF started to appear in lysosomes, and it showed maximal concentration within these organelles after 30 min. There were also signs of passage of PDGF through the Golgi complex, but only after 60 min. Treatment of the cells with chloroquine, a weak base that inhibits intralysosomal degradation, prevented disappearance of tracer from the lysosomes. The observations indicate that PDGF was internalized via coated regions of the plasma membrane and carried to lysosomes for degradation. Subsequent appearance of tracer within the Golgi complex could reflect receptor-ligand complexes that escaped degradation and were recirculated back to the cell surface.

Platelet-derived growth factor (PDGF) is a cationic 30,000-dalton protein with mitogenic activity for connective tissue and glial cells (1-3). Radioligand techniques have been used to demonstrate specific receptors for PDGF on fibroblasts, arterial smooth muscle, and glial cells (4-8). Incubation of the cells at 37°C resulted in degradation of PDGF. This was accompanied by a temporary loss in the ability to bind the factor, suggesting that the entire receptor-ligand complex had been internalized. The loss of radioactivity from the cells was inhibited by lysosomotropic weak bases such as chloroquine and methylamine, suggesting that the degradation took place in lysosomes (5, 7). Thus, subcellular localization of bound PDGF has so far been analyzed only by indirect methods. Clarification of this point is, however, essential for the further elucidation of the mechanism of action of PDGF. By using quantitative electron microscopic autoradiography and indirect immunofluorescence, we now present direct evidence for surface binding and intracellular processing of PDGF in human fibroblasts cultivated *in vitro*.

MATERIALS AND METHODS

Reagents. Human PDGF was purified to homogeneity (9). Biologically active, ^{125}I-labeled PDGF (^{125}I-PDGF) with a specific activity of about 20,000 cpm/ng was prepared as described (5). PDGF antibodies were produced in a rabbit and immunoglobulins were purified from the immune serum by chromatography on a column of staphylococcal protein A-Sepharose (Pharmacia) (10, 11).

Cells. Human foreskin fibroblasts (AG 1523) were obtained from The Human Genetic Mutant Cell Repository, Institute for Medical Research (Camden, NJ). Smooth muscle cells were isolated from the aorta of 8-month-old male Sprague-Dawley rats and used in the second passage (11). The cells were grown in medium F-12 with 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate (Hepes)/10 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonate (Tes) (pH 7.3), L-ascorbic acid at 50 μg/ml, gentamycin sulfate at 50 μg/ml, and 10% newborn calf serum at 37°C in an atmosphere of 5% CO_2 in air.

Electron Microscopic Autoradiography. Confluent cultures (35-mm Petri dishes) were cooled to 12°C and rinsed twice with medium MCDB 104 (GIBCO) containing 0.1% human serum albumin (binding medium). The cells were exposed to ^{125}I-PDGF at 50 ng/ml for 120 min at 12°C, rinsed five times, and incubated in binding medium at 37°C for 0, 5, 10, 30, 60, or 120 min. They were then fixed in 3% (wt/vol) glutaraldehyde in 0.1 M sodium cacodylate/HCl buffer (pH 7.3) with 0.05 M sucrose for 4 hr at 4°C, rinsed in buffer overnight, scraped off the Petri dishes, and transferred to plastic tubes. After postfixation in 1% cacodylate-buffered osmium tetroxide, the specimens were dehydrated, stained with 2% uranyl acetate in ethanol, and embedded in epoxy resin. Thin sections (80-90 nm) were cut on an LKB Ultrotome IV, collected on carbon-coated Formvar films, stained with lead citrate, and covered with a thin layer of carbon. Monolayers of Ilford L4 emulsion were applied to the sections by using the loop technique (12). After exposure at 4°C for 10-15 days, the autoradiographs were developed in Kodak D-19 developer (diluted 1:1) for 1.5 min at 22°C.

The grids were examined with a Philips EM 300 electron microscope. Quantitative analysis was restricted to human fibroblasts, on which biochemical studies have previously been performed (4, 5). For each time of incubation, four or five separate grids were used and 50-75 photographs of intact cells were taken sequentially to accumulate 200-300 developed grains. Micrographs were prepared at a final magnification of ×40,000. The location of the grains was determined by placing the smallest possible circle over them and recording the structural compartment hit by the center of the circle. The following compartments were used: (*i*) plasma membrane ± 2 HD [HD is half distance (90 nm); see ref. 13]; (*ii*) nucleus; (*iii*) endoplasmic reticulum; (*iv*) mitochondria; (*v*) vesicles + 1 HD; (*vi*) lysosomes (defined as vacuoles containing small vesicles, other inclusions, or both) + 1 HD; (*vii*) stacked Golgi cisternae + 1 HD; (*viii*) Golgi-associated vesicles + 1 HD; (*ix*) ground cytoplasm (in-

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Abbreviations: PDGF, platelet-derived growth factor; HD, half distance.

cluding cytoskeletal components and lipid droplets). The reason for including a rim of 2 HD around the plasma membrane and a rim of 1 HD around some intracellular organelles was to compensate for radiation spread from fine sources (13), in this case membranes. If HD rims of more than one structure covered the center of the circle placed over the grains, only the structure closest to the center was registered. Stereological measurements were performed to relate the percentage of grains in each compartment to the size of the latter. A test system consisting of an equilateral triangular network was superimposed on the micrographs and the volume density of the cellular compartments defined above was determined by point counting (14).

Indirect Immunofluorescence. Cultures were cooled to 12°C, rinsed twice with binding medium, exposed to PDGF at 50 ng/ml for 120 min at 12°C, rinsed another five times, and incubated in binding medium at 37°C for 0, 5, 10, or 15 min. The cells were fixed in 3% (wt/vol) formaldehyde in 0.2 M sodium phosphate buffer (pH 7.3) for 60 min at 22°C and rinsed three times for 5 min each at 4°C in binding medium. They were then exposed to PDGF antibodies at 100 μg/ml for 45 min at 4°C, rinsed five times, incubated with fluorescein-conjugated anti-rabbit IgG (Cappel Laboratories, Cochranville, PA; diluted 1:40) in phosphate-buffered saline for 45 min at 4°C, rinsed another five times, and mounted in glycerol. Cells not exposed to PDGF and cells incubated with immunoglobulins isolated from normal rabbit serum instead of PDGF antibodies served as controls. The specimens were examined in a Zeiss photomicroscope type III with epifluorescence illumination.

RESULTS

General Observations. Directly after binding of ^{125}I-PDGF to human foreskin fibroblasts at 12°C for 120 min (0 min), autoradiographic grains were located on the plasma membrane (Fig. 1 *a* and *b*). The specificity of binding was demonstrated by the ability of an excess of unlabeled PDGF to inhibit binding of labeled PDGF to these cells by more than 90%. In contrast, epidermal growth factor, fibroblast growth factor, and insulin did not interfere with the binding of labeled PDGF to the cells (4).

Warming the cells to 37°C resulted in internalization of radioactive material which, after 5–10 min, was concentrated to small peripheral vesicles (Fig. 1*c*) and subsequently transferred to lysosomes (Fig. 1*d*). At 60 min, grains were found also in the Golgi complex (Fig. 1*e*). The total amount of cell-associated radioactivity (determined in a gamma spectrometer) decreased rapidly with time, being only 20% of the initial value after 120 min at 37°C (data not shown; cf. ref. 5). The number of autoradiographic grains per cell similarly decreased with time.

Treatment of the cells with 20 μM chloroquine for 120 min at 37°C—i.e., during the warming-up period after exposure to ^{125}I-PDGF at 12°C—produced an increase in the size of the lysosomes and the number of vesicles and other inclusions present within them. Otherwise, no adverse effects on cell fine structure were noted. In contrast to the situation in untreated cells, large amounts of radioactive material remained in the lysosomes (Fig. 1*f*). Many grains were also found in the Golgi complex.

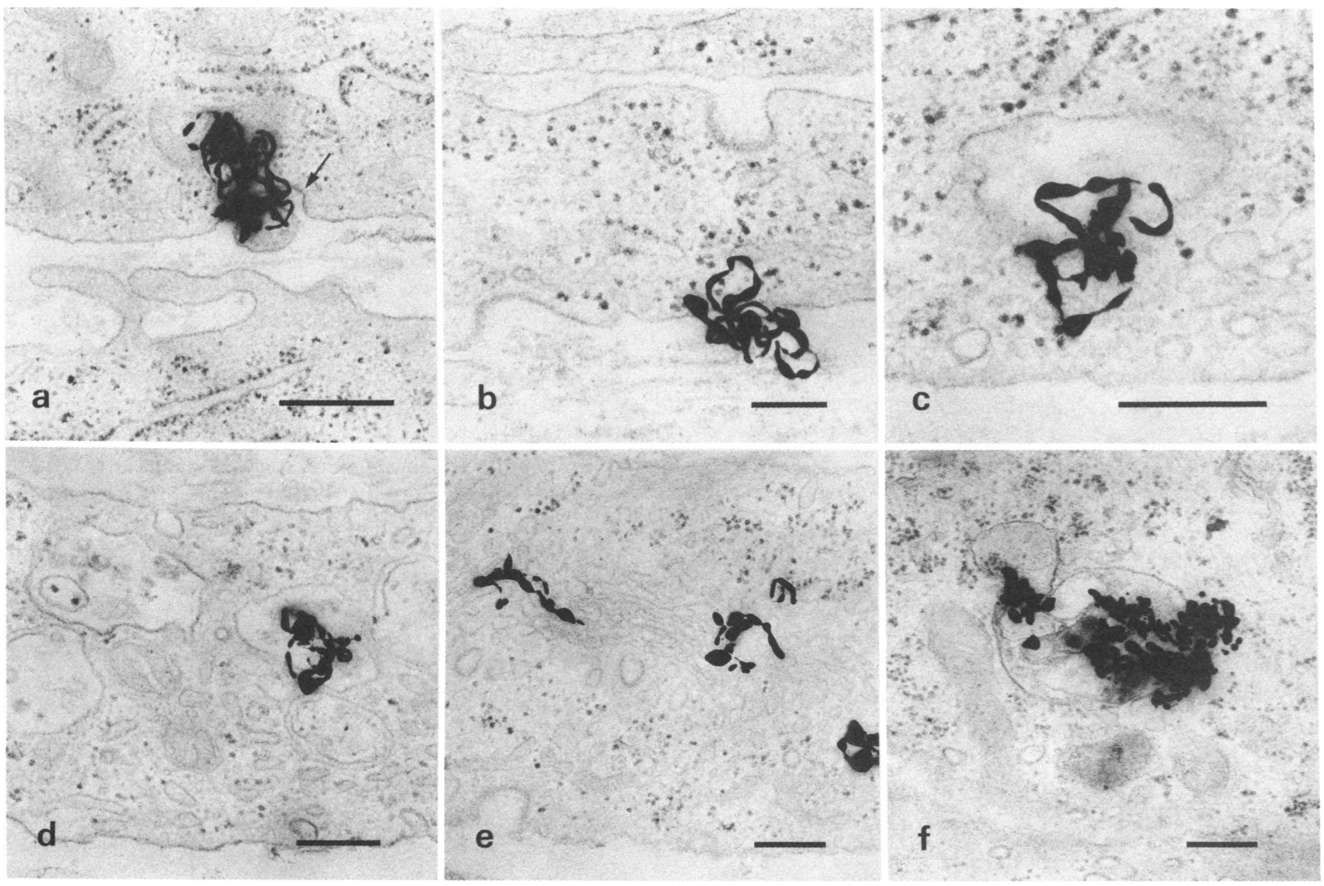

FIG. 1. Images of autoradiographic grains in human fibroblasts exposed to ^{125}I-PDGF at 12°C, rinsed, and incubated at 37°C for different times. Bars mark 0.25 μm. (*a* and *b*) Incubated 0 min at 37°C. Grains are associated with coated (*a*; arrow) and uncoated (*b*) regions of the plasma membrane. (*c*) Incubated 10 min at 37°C. Grains are associated with vesicles located just inside the plasma membrane. (*d*) Incubated 60 min at 37°C. Grains are associated with a lysosome. (*e*) Incubated 60 min at 37°C. Grains are associated with cisternae and vesicles of the Golgi complex. (*f*) Incubated 120 min at 37°C in the presence of 20 μM chloroquine. Numerous grains are associated with a lysosome.

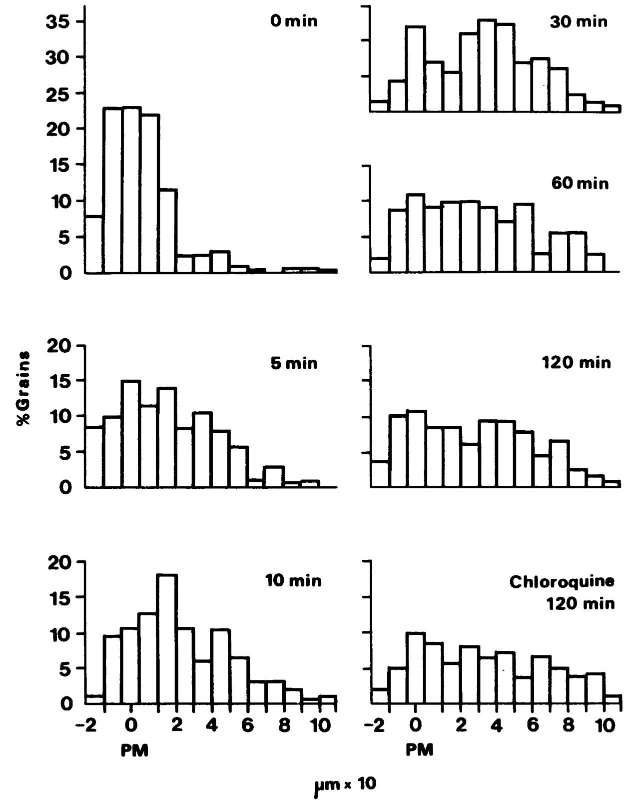

FIG. 2. Distribution of autoradiographic grains around the plasma membrane of human fibroblasts exposed to ^{125}I-PDGF at 12°C, rinsed, and incubated at 37°C for different times. The percentage of grains is plotted as a function of the distance of the grain center from the plasma membrane (PM).

Because the distribution of radioactive material changed with time and only parts of cells could be included on each plate, the frequency of occurrence of different organelles in the collected photographs could differ from one time to another. However, stereological measurements showed that this variation was small (data not shown). Chloroquine (120-min treatment) brought about a 40–45% increase in the volume density of lysosomes.

Comparative studies on rat arterial smooth muscle cells revealed results similar to those from human fibroblasts.

Table 1. Relationship of autoradiographic grains to coated regions of the plasma membrane in human fibroblasts

Incubation at 37°C, min	Surface-bound grains associated with coated regions of the plasma membrane, %	Coated regions of the plasma membrane, %
0	18.3	3.8
5	13.6	2.2
10	4.3	2.4
30	2.9	3.3
60	4.3	2.8
120	3.4	2.9
Chloroquine 120	4.8	1.6

All micrographs in the quantitative autoradiographic analysis were used. Surface-bound grains were defined as described in *Materials and Methods* and registered as associated with coated regions of the plasma membrane if located within 2 HD (180 nm) from them in the plane of the membrane. The coated portion of the plasma membrane was measured independent of the presence of grains.

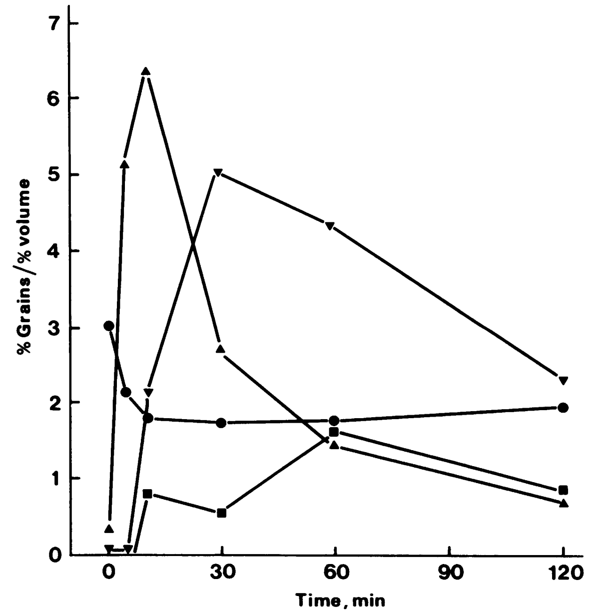

FIG. 3. Relationship of autoradiographic grains to cellular compartments in human fibroblasts exposed to ^{125}I-PDGF at 12°C, rinsed, and incubated at 37°C for different times. Those compartments in which signs of accumulation of radioactive material were obtained are shown (% grains/% volume > 1). ●, Plasma membrane; ▲, vesicles; ▼, lysosomes; ■, Golgi complex (stacked cisternae plus vesicles).

Surface Binding of ^{125}I-PDGF. Quantitative analysis (restricted to human fibroblasts) showed that 87% of the autoradiographic grains were associated with the plasma membrane directly after binding of ^{125}I-PDGF to the cells for 120 min at 12°C (Fig. 2). About 20% of these grains were associated with regions showing a fine bristle coat on the cytoplasmic side of the membrane. On the other hand, coated regions represented less than 4% of the surface of the cells (Table 1).

Uptake and Intracellular Transport of ^{125}I-PDGF. Surface-bound ^{125}I-PDGF was rapidly internalized after warming the cells to 37°C, although autoradiographic grains remained on the surface throughout the observation period (Figs. 2 and 3 and Table 2). At the same time, the percentage of surface-bound grains associated with coated regions of the plasma membrane decreased (Table 1). After 5–10 min, radioactive material was concentrated in small peripheral vesicles. Radioactivity started to appear in lysosomes after 10 min and at 30–60 min this was its main location. At 60 min radioactivity also appeared in stacked Golgi cisternae and Golgi vesicles. After 120 min, the relative amount of radioactive material in the lysosomes had decreased, although they were still the main site of radioactivity within the cells.

In cells allowed to bind ^{125}I-PDGF at 12°C and then treated with 20 μM chloroquine for 120 min at 37°C, the relative amount of radioactive material in the lysosomes was similar to that in untreated cells after 30–60 min (Table 2). Moreover, radioactivity remained within the Golgi complex.

Indirect Immunofluorescence. The immunofluorescence studies confirmed that PDGF was not randomly distributed after binding to the cell surface for 120 min at 12°C. Thus, a punctate pattern of fluorescent spots was obtained (Fig. 4*a*). Warming the cells to 37°C for 5–15 min resulted in loss of immunoreactivity from the cell surface (Fig. 4*b*). Similar observations were made on human fibroblasts and rat arterial smooth muscle cells. Controls were negative.

Table 2. Distribution of autoradiographic grains in human fibroblasts

Incubation at 37°C, min	Plasma membrane		Nucleus		Endoplasmic reticulum		Mitochondria		Vesicles		Lysosomes		Golgi cisternae		Golgi vesicles		Ground cytoplasm	
	%/%	No./vol	%/%	No./vol	%/%	No./vol	%/%	No./vol	%/%	No./vol	%/%	No./vol	%/%	No./vol	%/%	No./vol	%/%	No./vol
0	*2.98*	*8.44*	0.17	0.46	0.07	0.20	0	0	0.39	1.10	0.10	0.30	0	0	0	0	0.26	0.75
5	2.12	5.91	0.35	0.96	0	0	0	0	*5.12*	*14.20*	0.09	0.25	0	0	0	0	0.40	1.11
10	1.87	5.83	0.50	1.56	0.05	0.15	0	0	*6.39*	*20.05*	2.22	6.82	1.07	3.32	0.76	2.35	0.16	0.50
30	1.78	5.39	0.17	0.51	0	0	0.13	0.40	2.68	8.08	*4.97*	*14.99*	0.89	2.72	0.18	0.56	0.19	0.59
60	1.79	5.92	0.48	1.58	0	0	0.30	0.97	1.49	4.88	*4.32*	*14.21*	1.40	4.64	1.65	5.44	0.32	1.05
120	2.04	4.57	0.82	1.83	0.12	0.28	0.59	1.34	0.78	1.77	*2.33*	*5.20*	0.32	0.69	1.23	2.75	0.69	1.54
Chloroquine 120	1.87	5.50	0.23	0.69	0.10	0.30	0.25	0.73	0.53	1.57	*4.54*	*13.39*	1.44	4.42	1.35	3.94	0.31	0.91

Results are expressed as percentage of grains as related to percentage of volume (%/%) and as number of grains per unit volume (No./vol; arbitrary units). Italicized values indicate the site of maximal accumulation of radioactive material at the different times. Values greater than 1 indicate sites of location of radioactive material; 0 = no grains.

DISCUSSION

The results of the present electron microscopic autoradiographic and immunocytochemical study demonstrate that PDGF binds to the surface of human fibroblasts and rat arterial smooth muscle cells. It is then rapidly internalized via small vesicles and transferred to lysosomes for degradation. The observations confirm and extend earlier biochemical investigations (4–8) and indicate that the mode of uptake of PDGF into the cells is similar to that for other specific ligands, such as low density lipoprotein (15), epidermal growth factor (16, 17), insulin (18, 19), virus particles (20), growth hormone (21), α_2-macroglobulin (22), and lysosomal enzymes (23).

Directly after binding to the cells for 120 min at 12°C, PDGF was concentrated to coated regions of the plasma membrane. Warming to 37°C resulted in rapid internalization of PDGF, preferentially from these regions. Because the internalization is followed by a temporary loss of the ability of the cells to bind the ligand (5–7), the latter was probably ingested together with its receptor. It was then transported to lysosomes via small vesicles, usually lacking a membrane coat. The observations suggest that binding sites for PDGF were preclustered in coated regions on the cell surface or, alternatively, that the receptor–ligand complexes were able to move laterally within the plasma membrane and become trapped in coated regions at 12°C. The membrane coat was apparently lost early during the internalization process. Whether this took place before or after the endocytic vesicles were pinched off from the inner aspect of the plasma membrane could not be determined (cf. refs. 24 and 25).

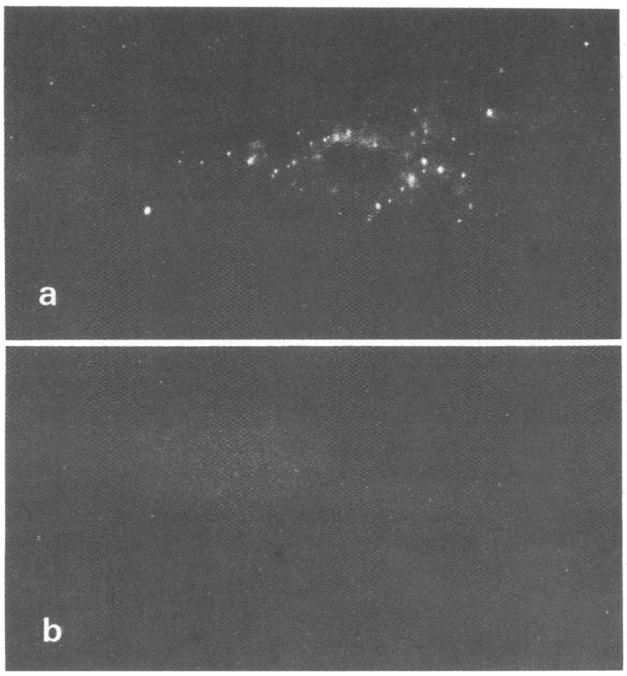

FIG. 4. Surface binding and internalization of PDGF in rat arterial smooth muscle cells as demonstrated by indirect immunofluorescence. (*a*) Directly after exposure to PDGF at 12°C. Reactive sites are distributed in a punctate pattern on the cell surface. (*b*) After 15 min at 37°C. Reactive sites have been removed from the cell surface and only weak background fluorescence is seen.

Transfer of PDGF to lysosomes was already seen after 10 min at 37°C, and this then remained the main location of the factor within the cells, with maximal activity after 30 min. There were also signs of passage of PDGF through the Golgi complex, but only after 60 min at 37°C. These observations suggest that the factor was carried directly to lysosomes and not via the Golgi complex. Thus, the intracellular transport of PDGF does not appear to follow the receptosomal pathway proposed in studies on other ligands (25). Tracer was found in both stacked cisternae and small associated vesicles of the Golgi complex. Because no acid hydrolase cytochemistry was performed, it cannot be excluded that a few of the latter may represent lysosomes.

The appearance of activity in the Golgi complex could possibly be a result of recirculation of a limited number of receptors (cf. ref. 5), assuming that smaller amounts of the factor escaped degradation in lysosomes and remained attached to the receptor. This would be in agreement with previous electron microscopic studies implicating the Golgi complex in recirculation of plasma membrane constituents (26).

Treatment of cells with chloroquine did not interfere with uptake and transport of ^{125}I-PDGF to lysosomes but did inhibit disappearance of radioactive material from them. This confirms earlier observations that weak bases prevent degradation of PDGF within the cells (5, 7). The mechanism of action is believed to involve accumulation of the base in lysosomes by permeation and proton trapping (27). This leads to a raised intralysosomal pH (28) and consequent inhibition of acid hydrolase activity.

After binding of ^{125}I-PDGF to the cells, and in spite of the rapid initial uptake at 37°C, a significant relative enrichment of radioactive material on the cell surface prevailed throughout 120 min of incubation. However, the total amount of cell-associated radioactivity decreased rapidly with time due to degradation of the factor.

After 10 min at 37°C and later, more than 95% of the surface-bound autoradiographic grains were associated with uncoated regions of the plasma membrane. One possible explanation of this finding is that small amounts of ^{125}I-PDGF were bound unspecifically to anionic glycoconjugates on the cell surface and that these, in contrast to the specific receptors, did not mediate uptake of the factor. Recirculation of a proportion of the receptor–ligand complexes could also contribute to the continuous labeling of the cell surface.

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